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Selective Targeting of Disease-Relevant Protein Binding Domains by *O*-Phosphorylated Natural Product Derivatives

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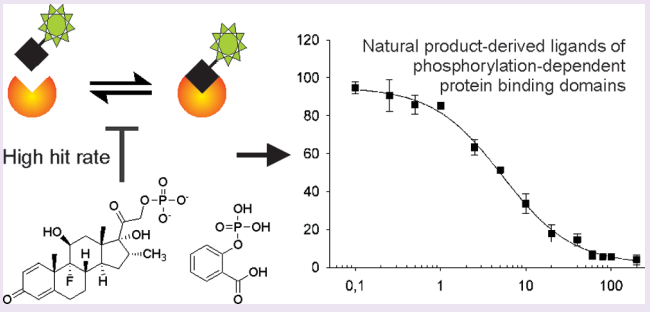
Supporting Information

ABSTRACT: Phosphorylation-dependent protein binding domains are crucially important for intracellular signaling pathways and thus highly relevant targets in chemical biology. By screening of chemical libraries against 12 structurally diverse phosphorylation-dependent protein binding domains, we have identified fosfosal and dexamethasone-21-phosphate as selective inhibitors of two antitumor targets: the SH2 domain of the transcription factor STAT5b and the substrate-binding domain of the peptidyl-prolyl isomerase Pin1, respectively. Both compounds are phosphate prodrugs with documented clinical use as anti-inflammatory agents in humans and were discovered with a high hit rate from a small subgroup within the screening library. Our study indicates *O*-phosphorylation of appropriately preselected natural products or natural product derivatives as a generally applicable strategy for the identification of non-reactive and non-peptidic ligands of phosphorylation-dependent protein binding domains. Moreover, our data indicate that it would be advisable to monitor the bioactivities of clinically used prodrugs in their uncleaved state against phosphorylation-dependent protein binding domains.



Protein−protein interactions form the basis of most biological processes.[1] Interactions between protein domains and peptide sequences bearing phosphorylated tyrosine, serine, or threonine residues (hereafter, phosphorylation-dependent protein−protein interactions) are crucial mediators of signaling through protein kinases and protein phosphatases, which is frequently dysregulated in human disease. This makes phosphorylation-dependent protein−protein interactions highly desirable targets for drug discovery and confronts chemists with the challenge of devising efficient starting points for related drug discovery projects. Nature, generally an excellent source for small-molecule lead structures,[2,3] has revealed only a small number of inhibitors of phosphorylation-dependent protein−protein interaction domains to date.[4] Moreover, most of these natural products comprise reactive chemical moieties and are thus thought to bear an increased risk of inducing nonspecific side effects. To explore possible design principles for non-peptidic and non-reactive inhibitors of phosphorylation-dependent protein−protein interactions, we screened known bioactive molecules against a structurally diverse panel of phosphorylation-dependent protein−protein interactions. This led to the identification of two *O*-phosphorylated natural product derivatives as selective, non-reactive inhibitors of phosphorylation-dependent protein binding domains. Fosfosal (Disdolen), the *O*-phosphorylated derivative of the natural product salicylic acid, and dexamethasone-21-phosphate, both of which have found clinical use as phosphate prodrugs against inflammatory diseases,[5,6] were identified as selective inhibitors of two protein domains considered to be antitumor targets: the SH2 domain of the transcription factor STAT5b and the substrate binding domain of the peptidyl-prolyl *cis/trans* isomerase Pin1, respectively. To our knowledge, this represents the first report of uncleaved phosphate prodrugs with prior clinical use that possess *in vitro* activity against phosphorylation-dependent protein binding domains.

To explore possible design principles for non-reactive inhibitors of phosphorylation-dependent protein−protein interactions, we tested small molecules with demonstrated affiliation to biologically relevant chemical space against a structurally diverse, 12-member panel of phosphorylation-dependent protein−protein interaction domains in fluorescence polarization assays. The assay panel consisted of phosphoserine/phosphothreonine-binding protein domains (the polo-box domains (PBD) of the

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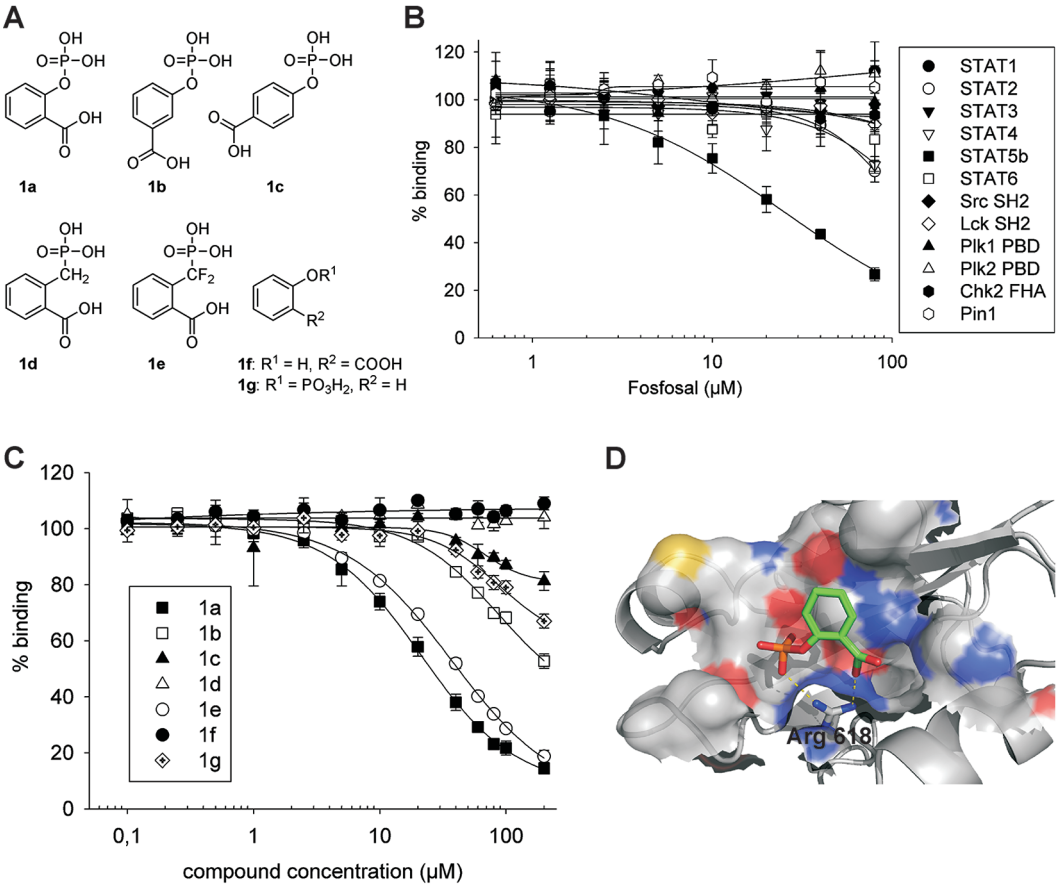

Figure 1. Fosfosal inhibits the STAT5b SH2 domain. (A) Chemical structures of fosfosal (**1a**) and derivatives **1b**−**1g** investigated in this study. (B) Activity and selectivity of fosfosal analyzed in fluorescence polarization assays. (C) Structure−activity relationships for fosfosal analyzed in fluorescence polarization assays. IC_{50} values were converted to K_i values as described.[36] (D) Binding mode of fosfosal to the STAT5b SH2 domain as suggested by molecular modeling. The graphic was generated using PyMOL.[37]

Ser/Thr kinases Plk1 and Plk2, the forkhead-associated (FHA) domain of the checkpoint kinase Chk2, and the substrate-binding domain of the peptidyl-prolyl *cis/trans* isomerase Pin1) and phosphotyrosine-binding protein domains (the SH2 domains of the transcription factors STAT1, STAT2, STAT3, STAT4, STAT5b, and STAT6, as well as the SH2 domains of the tyrosine kinases Src and Lck).[4,7] Five chemical libraries comprising a total of 5019 known biologically active small molecules (a large proportion of which are natural products or natural product derivatives) were screened in all 12 assays at a concentration of approximately 50 μM. Compounds were defined as hits if they selectively inhibited one of the targets by at least 50% at this concentration in duplicate screens. To approach our goal of identifying non-reactive small molecules, retesting of the screening hits was performed in the presence or absence of dithiothreitol (DTT). This nucleophile is frequently used as reducing agent in biochemical assays and has been shown to decrease the activity of compounds bearing Michael acceptor systems.[8,9] Furthermore, we investigated whether the compounds' inhibitory effect increased over time, since time-dependent inhibition is a hallmark of reactive compounds. Only selective inhibitors with activities found to be independent of the exposure to DTT, and the time of the incubation with the respective protein domains, were analyzed in more detail in this study. This led to the identification of two substances, both of which have found clinical use as phosphate prodrugs against inflammatory diseases. First, fosfosal (Disdolen) (**1a**), the phosphate prodrug of the natural product salicylic acid, was found to selectively target the SH2 domain of the transcription factor STAT5b (K_i = 17.4 ± 0.6 μM) (Figure 1A,B and Supplementary Figure S1). Second, dexamethasone-21-phosphate (**2a**), a close derivative of the natural product cortisol, selectively bound to the substrate binding domain of the peptidyl-prolyl *cis/trans* isomerase Pin1 (K_i = 2.2 ± 0.1 μM) (Figure 2A,B and Supplementary Figure S4).

The chemical structure of fosfosal (2-(phosphonooxy)-benzoic acid, **1a**) was confirmed by resynthesis (Supporting Experimental Procedures and Supplementary Figure S2). Inhibitory activity against STAT5b strictly required the phosphate group, evidenced by the complete inactivity of salicylic acid (**1f**) (Figure 1A,C). Deletion of the carboxy group as represented by phenyl phosphoric acid (**1g**) caused a strong yet incomplete reduction of activity (Figure 1A,C). To understand the binding mode of fosfosal, we performed homology modeling of the STAT5b SH2 domain based on the X-ray structure of the highly related STAT5a protein (93% sequence identity).[10] Docking experiments using AutoDock Vina[11] suggested that fosfosal may occupy the phosphotyrosine binding pocket of the STAT5b SH2 domain (Figure 1D) similar to the binding mode of a phosphotyrosine-containing peptide to the SH2 domain of STAT1 (Supplementary Figure S3).[12] Arg618 at the bottom of the STAT5b

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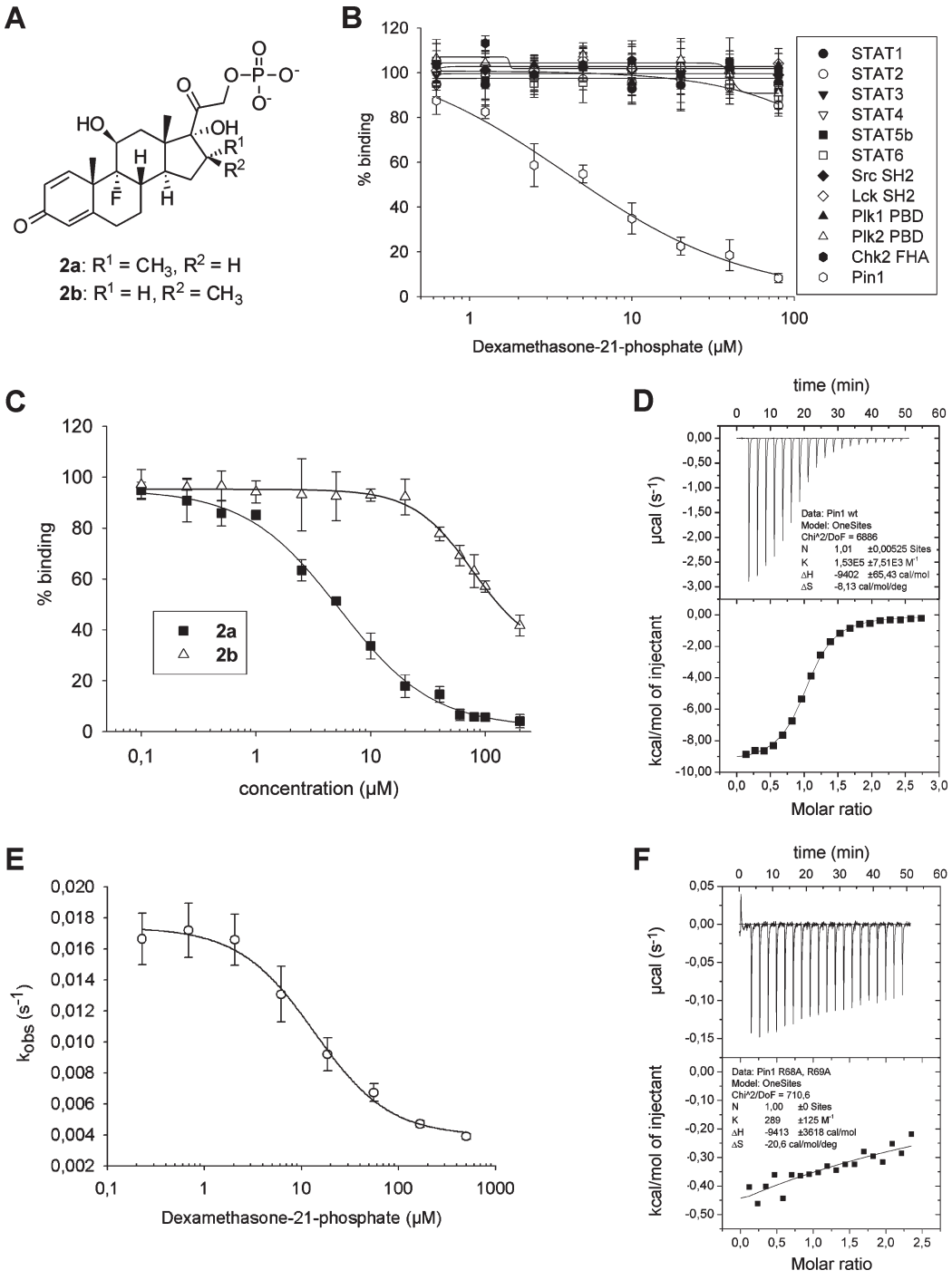

Figure 2. Dexamethasone-21-phosphate selectively inhibits Pin1. (A) Structures of dexamethasone-21-phosphate (**2a**) and its C-16 epimer betamethasone-21-phosphate (**2b**). (B) Activity and selectivity of dexamethasone-21-phosphate analyzed in fluorescence polarization assays. (C) Activities of dexamethasone-21-phosphate and betamethasone-21-phosphate against Pin1 in fluorescence polarization assays. IC_{50} values were converted to K_i values as described.[36] (D) Binding of dexamethasone-21-phosphate to full-length Pin1 as analyzed by ITC. (E) Dexamethasone-21-phosphate inhibits *cis*/*trans* isomerization of the Pin1 substrate Suc-AEPF-pNA. (F) Dexamethasone-21-phosphate displays only weak binding to a Pin1 Arg68, 69Ala double point mutant as analyzed by ITC.

SH2 domain was predicted to form an electrostatic interaction with both the phosphate and the carboxy group of fosfosal. Since STAT5b Arg618 is critically required for binding of the fluorescein-labeled, phosphotyrosine-containing peptide used in the fluorescence polarization-based binding assay, experimental validation of this binding pose by testing point mutants was unfeasible. Validation of the suggested binding mode by comparing the affinities of fosfosal for wild-type STAT5b, and point mutants (e.g., Arg618Ala or Arg618Lys), respectively, by isothermal titration calorimetry (ITC) was prevented by insufficient protein solubility, which did not allow for quantitative determination of the affinity between fosfosal and wild-type STAT5b.

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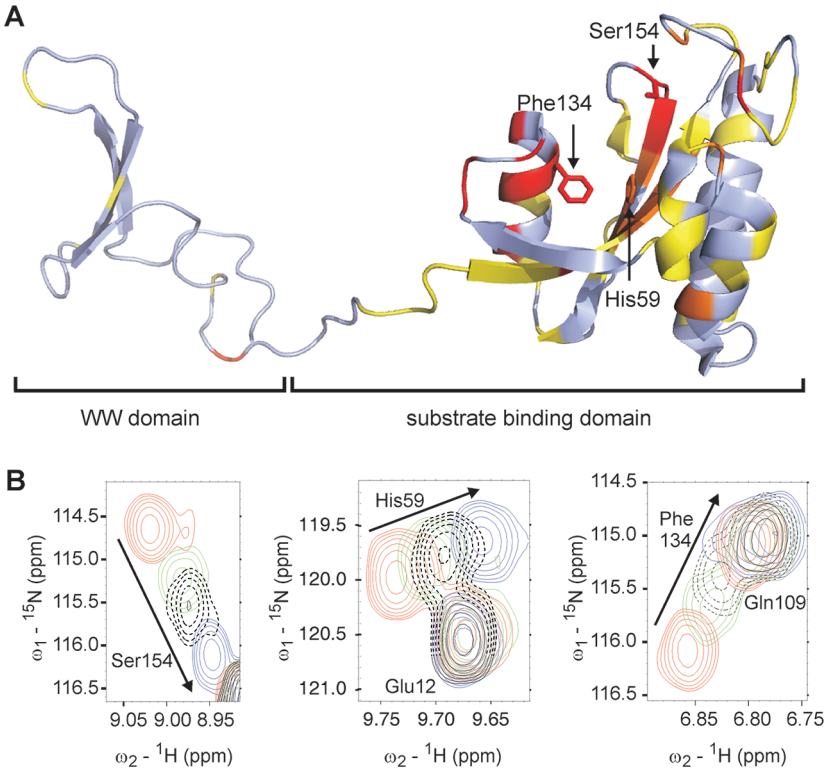

Figure 3. Dexamethasone-21-phosphate **2a** binds to the substrate binding domain of Pin1. (A) Graphic representation of the chemical shifts in the $^{1}H,^{15}N$-HSQC-NMR of full-length Pin1 (300 μM) observed after addition of **2a** (600 μM). The shifts are superimposed onto the solution structure of Pin1 (PBD code: 1NMV).[17] Color code: red, very strong shift ($\Delta\delta \geq 0.125$ ppm); orange, strong shift (0.075 ppm $\leq \Delta\delta < 0.125$ ppm); yellow, medium shift (0.025 $\leq \Delta\delta < 0.075$ ppm); light blue, weak shift ($\Delta\delta < 0.025$ ppm). The graphic was generated using PyMOL.[37] (B) The amide resonances of Ser154, His59, and Phe134 in ^{15}N-labeled Pin1 (300 μM) in the absence of **2a** (red) show strong and dose-dependent chemical shifts upon addition of **2a** (150 μM, green; 300 μM, dotted black; 600 μM, blue). The graphic was generated using Sparky.[38]

Instead, we synthesized 3-(phosphonooxy)benzoic acid (**1b**) and 4-(phosphonooxy)benzoic acid (**1c**) (Supporting Experimental Procedures) and observed decreasing activities with increasing distances between the phosphate and the carboxylate groups (Figure 1A,C), which is consistent with the observed structure−activity relationships.

Formal substitution of the phosphate ester bridging oxygen by a CH_2 group or a CF_2 group to yield methylphosphonates and difluoromethylphosphonates, respectively, is a frequently applied synthetic strategy by which to prevent enzymatic cleavage of phosphate esters.[13] We synthesized both the methylphosphonate (**1d**) and the difluoromethylphosphonate (**1e**) corresponding to fosfosal (Figure 1A and Supporting Experimental Procedures). While the methylphosphonate (**1d**) was inactive, activity of the difluoromethylphosphonate (**1e**) was decreased by only less than 2-fold (K_i = 26.2 ± 3.3 μM) compared to fosfosal (Figure 1C), demonstrating the general feasibility of converting phosphate esters targeting the STAT5b SH2 domain to stabilized derivatives while retaining target affinity. Together with chromone-based acylhydrazones,[14,15] fosfosal (**1a**) and its phosphatase-stable derivative **1e** are among the first and most active non-peptidic inhibitors of the interaction between the SH2 domain of unphosphorylated STAT5b and its native peptide binding motif *in vitro*.

Structure−activity relationships for binding of dexamethasone-21-phosphate (**2a**) to Pin1 also demonstrated the phosphate group to be of crucial importance for binding (Figure 2A, C), because both dexamethasone (**2c**) and dexamethasone-21-acetate (**2d**) were almost completely inactive in the fluorescence polarization assay (Supplementary Figure S5). Of note, the C-16 epimer betamethasone-21-phosphate (**2b**) displayed approximately 35-fold lower activity than dexamethasone-21-phosphate (**2a**) (K_i values: **2a**, 2.2 ± 0.1 μM; **2b**, 76.8 ± 15.9 μM), indicating selective recognition of **2a** by Pin1 (Figure 2A,C). The epimers' relative activities in the fluorescence polarization-based, indirect binding assay were reflected by their significantly different dissociation constants against full-length Pin1 as determined by isothermal calorimetry titrations (ITC) (K_d values: **2a**, 6.8 ± 0.2 μM; **2b**, 542 ± 81 μM) (Figure 2D and Supplementary Figure S6A). The relative activities of **2a** and **2b** against Pin1 in the latter two assays were confirmed by their strongly divergent inhibitory effects in a Pin1 isomerase assay (**2a**, EC_{50} = 14.4 ± 1.3 μM; **2b**, 26% inhibition at 500 μM) (Figure 2E and Supplementary Figure S6B).[16]

Apart from the substrate binding domain, Pin1 contains a second domain with potential affinity to pSer-Pro motifs, the WW domain. To analyze whether dexamethasone-21-phosphate also binds to the WW domain, we performed ITC experiments on the separate WW domain and the substrate binding domain of Pin1, which demonstrated a clear preference of the molecule for the substrate binding domain (K_d = 9.2 ± 1.1 μM) over the WW domain (K_d = 796 ± 282 μM) (Supplementary Figure S7). This preference was confirmed by $^{1}H,^{15}N$-HSQC-NMR using ^{15}N-labeled full-length Pin1, which showed numerous strong shifts in the substrate binding domain (Figure 3A and Supplementary Figure S8).[17] Amino acids with strong and dose-dependent shifts of their amide resonances include Ser154 and His59, which

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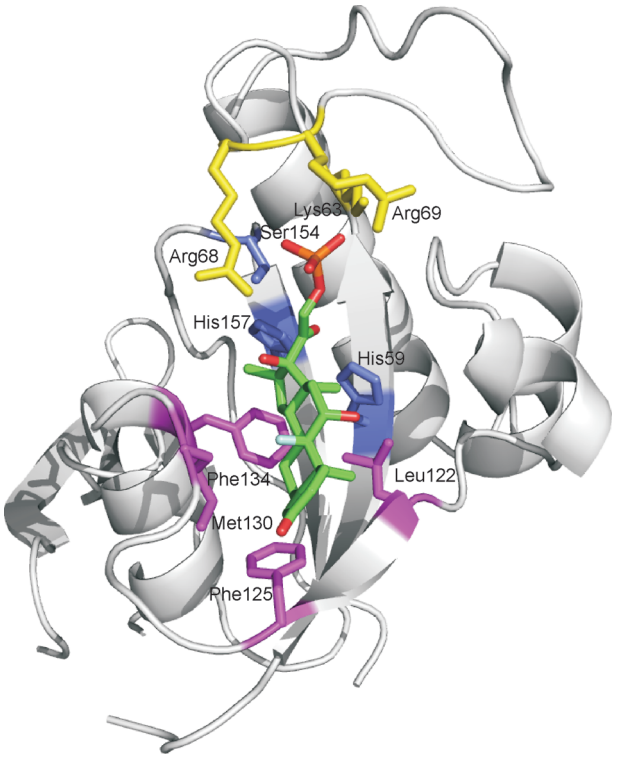

Figure 4. X-ray structure of the complex of Pin1 (Arg14Ala mutant) and dexamethasone-21-phosphate **2a** (PDB code 3TC5). Amino acids are color-coded based on their functional contribution to binding of **2a**. Yellow, phosphate binding; light blue, exocyclic carbonyl oxygen binding; magenta, hydrophobic core and C-16 methyl group binding. The graphic was generated using PyMOL.[37]

interact with the carbonyl oxygen of the peptide bond to be isomerized by the enzyme (Figure 3B).[18] Similarly, Phe134 at the bottom of the hydrophobic proline binding pocket also displayed strong shifts in the presence of **2a**. In contrast, fewer and weaker shifts of the backbone amide resonances were observed in the WW domain upon addition of **2a**. Consistent with its weaker affinity for Pin1, the epimer betamethasone-21-phosphate (**2b**) showed less and weaker shifts in the $^{1}H,^{15}N$-HSQC-NMR (Supplementary Figure S9). To further elucidate the binding mode of dexamethasone-21-phosphate within the substrate binding domain, we mutated two crucial arginine residues in the phosphate binding pocket of Pin1 to alanine.[19] The Pin1 Arg68, 69Ala double mutant bound to dexamethasone-21-phosphate with only very weak affinity in isothermal calorimetry titrations ($K_d = 4.4 \pm 0.9$ mM) (Figure 2F), indicating that the compound occupies the phosphoserine-binding pocket of the substrate binding domain.

To analyze the molecular basis for the interaction between dexamethasone-21-phosphate (**2a**) and Pin1 in more detail, we soaked **2a** into crystals of the Pin1 Arg14Ala mutant, which had previously been shown to display favorable crystallization properties while retaining the binding functionality of the wild-type enzyme.[18,20] Crystallographic analysis of Pin1 bound to **2a** confirmed the localization of the ligand's phosphate group to the positive patch in the Pin1 substrate binding domain formed by Lys63, Arg68, and Arg69 (Figure 4 and Supplementary Figure S10A), consistent with the ITC data (Figure 2F). The keto group in the ligand's side chain is located to a hydrophilic pocket comprised of Ser154, His59, and His157 (Figure 4 and Supplementary Figure S10B). The methyl group attached to C-16, the spatial orientation of which solely accounts for the activity difference between **2a** and **2b**, is appropriately positioned for a hydrophobic interaction with Phe134. The more hydrophobic side of the inhibitor's tetracyclic ring system is accommodated by the hydrophobic pocket composed of Phe134, Leu122, and Met130, which form the binding pocket for the proline ring in Pin1 substrates,[21] and extends to Phe125.

Dexamethasone-21-phosphate (**2a**) is among the few non-peptidic and non-reactive inhibitors of Pin1 with activities below 10 μM, along with aryl indanyl ketones,[22] phenyl-imidazole-based compounds,[23] quinazoline derivatives,[24] and tetraoxobenzophenanthrolines.[25] Importantly, **2a** is the only member of this group whose specificity was tested against a panel of related phosphorylation-dependent protein binding domains and which thus can be regarded as Pin1-specific with regards to this class of protein domains. The Pin1 inhibitor juglone is thought to interact covalently with the enzyme[26] and has been reported to exert additional biological activities.[27] While a number of non-reactive ligands for the substrate-binding domain/enzymatic domain of Pin1 with submicromolar or nanomolar affinities have been described, these agents comprise either peptides[18,28] or peptide mimetics.[27,29−31]

Retrospective analysis of the composition of the screening library revealed that both **1a** and **2a** are members of the very small subgroup of screening compounds tested in this study that are represented by natural products or natural product derivatives bearing *O*-phosphorylated hydroxyphenyl or hydroxymethyl groups (12/5019 compounds (0.24%), see Supplementary Figure S11) and hence display structural similarity to the side chains of phosphotyrosine and phosphoserine, respectively. This subgroup has yielded selective, non-reactive inhibitors of phosphorylation-dependent protein binding domains with a validated hit rate of 1.4% in this study (2 hits from a subgroup of 12 compounds against 12 targets), a ~200-fold increase over the validated hit rates of approximately 0.006−0.009% we had previously obtained in screens using unbiased, chemically diverse chemical libraries against phosphorylation-dependent protein−protein interactions.[7−9,14] No other compound from the remaining 5007 compounds was discovered in this screen that satisfied our criteria of selectivity and time-independent inhibition.

In summary, we have identified two phosphate prodrugs with documented clinical use in humans,[5,6] the natural product derivatives fosfosal (**1a**) and dexamethasone-21-phosphate (**2a**), as selective, non-reactive inhibitors of two antitumor targets: the STAT5b SH2 domain and the Pin1 substrate binding domain, respectively. To our knowledge, this represents the first report of uncleaved phosphate prodrugs with prior clinical use to possess *in vitro* activity against phosphorylation-dependent protein binding domains. Activities, selectivities, and structure−activity relationships of **1a** and **2a** were characterized by a combination of biochemical methods and organic synthesis. The binding modes of the compounds were studied by a combination of X-ray crystallography, NMR spectroscopy, and molecular docking, and indicated that the compounds occupy the respective proteins' ligand-binding domain. Our data indicates *O*-phosphorylation of appropriately preselected natural products or natural product derivatives as a widely applicable and intuitive method by which to generate non-peptidic lead structures for phosphorylation-dependent protein binding domains. This strategy should be applicable to the large number of phosphorylation-

dependent protein binding domains known to date. In addition, large collections of natural products or derivatives thereof comprising suitable aliphatic or aromatic hydroxyl groups can be converted to phosphate esters. This could provide an abundant source of non-peptidic and non-reactive molecules targeting phosphorylation-dependent protein binding domains. Successful implementation of this approach would represent a significant step toward approaching chemical space suitable for targeting phosphorylation-dependent protein−protein interactions. The phosphate esters identified by the approach suggested here can be developed to hydrolytically stable phosphate mimics,[13] as was demonstrated by converting fosfosal (**1a**) into its difluoromethylphosphonate derivative **1e** (Figure 1A,C). Subsequent introduction of protecting groups that mask the phosphonate's negative charges and are prone to intracellular hydrolysis can generate cell-permeable compounds, as was impressively shown in a number of recent studies.[32−35] Efforts to develop fosfosal and dexamethasone-21-phosphate into highly potent agents suitable for use in whole cells are currently ongoing and will be reported in the near future. On a wider note, our data indicate that it would be advisable to analyze the activities of uncleaved phosphate prodrugs intended for clinical use with respect to phosphorylation-dependent protein binding domains.

METHODS

Details on the chemical synthesis, the chemical libraries, plasmid construction, assay development and all biophysical techniques are summarized in the Supporting Information.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

Accession Codes

Coordinates for **2a** bound to Pin1 have bzeen deposited in the Protein Data Bank under accession code 3TC5.

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